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Alex Hopke[1^], Alistair J. P. Brown[2], Rebecca A. Hall[3], Robert T. Wheeler[1,4,$]

[1]Molecular & Biomedical Sciences, University of Maine, Orono, ME, USA

[2]MRC Centre for Medical Mycology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, UK

[3]Institute of Microbiology and Infection, and School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK

[4]Graduate School of Biomedical Sciences and Engineering, University of Maine, Orono, ME, USA

[^]Current address: BioMEMS Resource Center, Division of Surgery, Innovation and Bioengineering, Department of Surgery, Massachusetts General Hospital, Shriners Burns Hospital, Harvard Medical School, Charlestown, MA, USA

[$]Correspondence to:
Robert T. Wheeler
University of Maine
Orono, ME, 04473
USA
robert.wheeler1@maine.edu

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Abstract

Modern medical advancements and the HIV/AIDS pandemic have led to an at-risk immunocompromised population and a concomitant rise in deadly infections from opportunistic fungi. The polysaccharide cell wall plays an outsized role in fungal pathogenesis and therapy, because it acts as both an environmental barrier and as the major interface with the host immune system. Human fungal pathogens use architectural strategies to mask epitopes from the host and prevent immune surveillance, and recent work elucidates how biotic and abiotic stresses present during infection can either block or enhance masking. The signaling components implicated in regulating fungal immune recognition can teach us how cell wall dynamics are controlled, and represent potential targets for interventions designed to boost or dampen immunity.

The fungal cell wall, an Achilles heel for a destructive class of pathogens

Recent years have seen the emergence of fungi as a major threat to public health and food security. The success of modern medical advancements like chemotherapy and organ transplantation extend patient life, but leave immune compromised patients susceptible to invasive infections by opportunistic fungal pathogens like *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*. These invasive fungal infections are accompanied by unacceptably high mortality rates, despite modern antifungal therapies. *Candida*, as an example, is the 4th most common cause of nosocomial bloodstream infections, thought to cause greater than 400,000 life-threatening infections annually [1]. Beyond being a threat to public health, fungi threaten agriculture, destroy crops, devastate amphibian and bat species, and are responsible for major extinctions caused by infectious disease [2]. Facing a global assault on plants, animals and our own health, we must devise novel strategies to address fungal infections.

Opportunistic fungi, like other pathogens in this class, are able to thrive in challenging and rapidly changing environments by using multiple pathways for stress adaptation [3]. For *C. albicans*, these stresses include environmental challenges, drug exposure and immune attack. Recent work has shown that the fungal responses to these stresses include altering their cell surfaces to enhance or limit immune recognition and responses, linking the two disparate fields of **cell wall integrity** (see Glossary) and immunity (Fig. 1, Key Figure). This new work highlights the translational importance of the cell wall, which serves as an effective drug target against both bacteria and fungi, and includes essential microbial components recognized by conserved innate immune pattern recognition receptors (**PRRs**). In this review, we summarize our current knowledge of fungal cell wall remodeling in response to the environment, with a focus on responses to stress relevant to infection. We also highlight the role of fungal cell wall remodeling in epitope masking.

Adaptation to stresses during infection: fungal "feng shui"

Normal fungal cell wall architecture and stress responses.

The cell wall is a versatile organelle that plays a central role in the maintenance of cellular integrity. It is both the primary barrier against the external environment and the initial point of contact between the host and fungus, where it initiates immune responses via immune receptors.

Most pathogenic fungi have an inner cell wall layer of **chitin** and **β-glucan** which provides cellular integrity, but the outer layer composition of the cell wall varies among fungal species [4]. In *C. albicans*, the outer layer consists of mannosylated glycoproteins connected to the inner layer by modified GPI anchors and (1,6)-β-glucan linkages to the inner core of β-glucan and chitin. This cell wall is modified between yeast and hyphal forms [5]. *A. fumigatus* also has different layers that vary with morphology, as conidia have a layer of α-glucan along with melanin and hydrophobic rodlets in their outer cell wall, while hyphae have α-glucan, galactomannan and galactosaminoglycan. Similarly, *Histoplasma capsulatum* and other dimorphic fungi are covered with α-glucan during infection. In contrast, *C. neoformans* has a thick outer capsule made largely of glucuronoxylomannan and galactoxylomannan attached to α-glucan.

Although we know that the cell wall is dynamic, we still understand little about the pathways that regulate its architecture in pathogenic fungi. In the model fungus *Saccharomyces cerevisiae*, the cell wall integrity signaling network is able to sense stresses and transduce them into both transcriptional and post-transcriptional changes that modulate cell wall structure [6, 7]. This signaling network, which is evolutionarily conserved in *C. albicans* and other fungi, is initiated by stress sensors for pH, oxygen, carbon dioxide, shifts in carbon sources, osmotic shifts, reactive oxygen, nitrogen, and sulfur species, temperature, and direct cell wall perturbation [8-14]. Important parts of this network include cell wall sensors, two-component signaling proteins, MAPK signaling components, protein kinase C, calcineurin, transcription factors, and cytosolic and cell wall effectors [15]. Fungi use these signals to maintain "feng shui", rapidly and accurately remodeling the architecture and composition of the cell wall to minimize the impact of these stressors in an ever-changing environment.

Cell wall stresses are felt during infection

A living host presents unique niches that require fungi to rapidly adapt their cell wall. Recent work has addressed how fungi respond to single and combinatorial stresses, and how cell wall integrity responses are activated *in vivo*. We know that changes in carbon availability, pH, oxygen availability and immune attack are all encountered *in vivo* [4, 16-18]. Rigorous in vitro experiments link these stresses to cell wall changes and altered immune recognition, and it is

clear that immune attack also regulates cell wall remodeling and immune recognition *in vivo* [19-24].

In addition to natural environmental stresses, the echinocandin class of antifungal drugs imposes severe stress on the fungal cell wall by inhibiting β-glucan synthase. Exposure to echinocandins has been shown to induce numerous changes to the fungal cell wall, including lower β-glucan content, unmasking of cell wall β-glucan, and increased chitin synthesis and exposure [25-29]. Importantly, these responses are different *in vivo* and *in vitro,* indicating that signaling pathways may be activated or repressed by other cues within host niches [30].

The host environment also presents the threat of multiple stressors. *In vitro* work on multiple stressors suggests that *C. albicans* uses microbial adaptive prediction, where exposure to an initial stress can influence survival when encountering a later stress [3]. These data emphasize the limits of a reductionist approach and the necessity to study fungal physiology during infection.

Candida *stress pathways are regulated during infection and important for pathogenesis*

For *C. albicans*, the importance of many cell wall genes for fungal viability in the host has been confirmed through deletion mutants. For instance, conditional repression of ***FKS1*** in a mouse model of disseminated candidiasis confirms its requirement *in vivo* [31]. ***PHR1***, which remodels cell wall β-glucan and has been demonstrated to be recruited to sites of neutrophil attack, is required for virulence during disseminated but not vulvovaginal candidiasis [32]. This finding of niche-specificity of cell wall requirements and virulence was powerfully generalized using barcoded mutants [33]. Similar *in vivo* competition experiments have more recently demonstrated a connection between cell wall epitope exposure and gastrointestinal fitness [34].

Tools such as **Nanostring profiling**, **RNAseq** and cell intrinsic reporters have also enabled investigators to probe fungal physiology during infection [30, 35-38]. Imaging of single-cell responses using fluorescent reporters has also been useful in both endpoint and intravital studies using mice and zebrafish hosts [35, 37, 38]. Significantly, Nanostring profiling of fungal gene transcripts during drug treatment of disseminated candidiasis has demonstrated that the transcriptomic responses to caspofungin differ significantly between *in vivo* and *in vitro* conditions [30]. *In vivo* profiling has also revealed that transcripts related to the fungal cell wall

and remodeling can differ between *C. albicans* strains and are impacted by the immune status of the host [27]. These studies emphasize that we now have the tools to translate *in vitro* findings into an understanding of cell wall remodeling during infection.

Pathogen recognition and epitope masking: wielding the cell wall as a mask

Fungal structures are recognized by pattern recognition receptors

The innate immune system is constantly on alert for microbial pathogens. To achieve this, the host uses a vast array of pattern recognition receptors (PRRs), which are capable of recognizing distinct components not normally found in the body. Upon binding these foreign ligands, called pathogen associated molecular patterns (**PAMPs**), PRRs initiate signaling cascades that activate downstream responses including phagocytosis, microbial killing mechanisms and cytokine production.

Fungal cell wall PAMPs include chitin, mannan and β-glucan, which are recognized by host innate immune PRRs that include multiple Toll-like receptors and C-type lectin receptors (**CLRs**) [39-41]. CLRs are particularly important for antifungal immunity, as evidenced by susceptibility to fungal infection in patients lacking effective CLR activity [42]. The cell wall, by controlling both fungal viability and host responses, therefore serves as a major focal point in determining the outcome of fungal infection.

Animal and plant pathogens mask epitopes from immune recognition

Manipulation of cell wall architecture to mask epitopes from host recognition is actually quite widespread and has been observed during fungal infection of plants and insects [43-46]. Importantly, epitope masking is also seen in multiple fungi capable of infecting humans.

In particular, fungi seem to go to great lengths to mask β-glucans. *H. capsulatum* masks its β-glucan with α-glucan and *Paracoccidioides brasiliensis* actually converts its (1,3)-β-glucans to (1,3)-α-glucan in the host [47, 48]. *A. fumigatus* also masks β-glucan, though this PAMP becomes exposed during germination or in hypoxic host environments [23, 49]. This basal β-glucan masking in *Aspergillus* depends on cell wall architecture and involves the hydrophobin RodA, α-glucan, galactosaminogalactan and the pigment protein PskP [50-53]. Interestingly, *A. fumigatus* RodA also appears important for blocking Dectin-2 mediated host responses,

presumably to mannan epitopes [51]. *Fonsecaea pedrosoi*, a major agent of chromoblastomycosis, masks β-glucan in the cell wall of its sclerotic cells with a chitin-like component [54]. *C. neoformans* masks PAMPs, including β-glucan, behind a thick capsule [55]. *C. albicans* also masks its β-glucan from **Dectin-1**, with limited exposure at places like bud scars. It is generally thought that the outer layer of mannan and mannoproteins in the cell wall shields the inner β-glucan from Dectin-1 recognition [4]. These diverse outer cell wall layers likely reflect species-specific evolutionary solutions to deal with environmental stresses, but they all function to restrict host access to the inner cell wall.

Maintenance of β-glucan masking by *C. albicans* requires a complex and diverse set of pathways in fungi. Disruptions of mannose structure, GPI anchor synthesis, phosphotidylserine biosynthesis, cell wall integrity signaling, β-glucan remodeling and β-glucan synthesis have all been shown to result in β-glucan unmasking [29, 56-62]. Atomic force microscopy demonstrates that β-glucan unmasking correlates with the increased surface roughness [63], while super-resolution microscopy has characterized the fine-scale architecture of caspofungin induced β-glucan unmasking in *C. albicans* [64]. Importantly, in each of these cases, the exposure of β-glucan drives enhanced Dectin-1 recognition and inflammatory responses against fungi. While epitope unmasking clearly impacts interactions with host immunity, the cell wall dynamics involved in the maintenance of epitope masking are only beginning to be understood.

Biotic and abiotic stresses cause epitope masking and unmasking: timely wearing the mask

Changes in the fungal environment trigger changes in epitope exposure

Adaptation to the multitude of environmental cues in the host incuses morphogenesis and activates stress response pathways. However, host-derived conditions, antifungals and host immune action have also been shown to induce considerable cell wall remodeling in fungi [65]. These changes in the fungal cell surface have profound effects on how the invading pathogen is perceived by the innate immune system. Three recent studies extend this previous work to demonstrate how fungal adaptation to the host niche influences the innate immune response.

Acidic pH enhances glucan unmasking

One key parameter *C. albicans* experiences during colonisation of the vaginal mucosa is low mucosal pH, with the vaginal mucosa being between pH 4-5, depending on age, ethnic origin and

position in the menstrual cycle [66]. Investigation of the effect of pH adaptation on *C. albicans* identified that exposure to acidic environments induces the exposure of β-glucan and chitin, two cell wall carbohydrate PAMPs which are normally buried beneath the mannan fibrils [24] (Fig. 2). This exposure of underlying cell wall carbohydrates has been termed cell wall unmasking [29]. Both chitin and β-glucan are recognised by the innate immune system [67, 68], and *C. albicans* cells that have adapted to acidic conditions elicited a strong pro-inflammatory innate immune response, and enhanced neutrophil recruitment [24], immune responses characteristic of genital thrush.

Currently, the precise structural rearrangements that result in this cell wall unmasking are unknown. Visualisation of the ultrastructure of *C. albicans* revealed that the mannan fibril layer was reduced upon adapted to acidic conditions [24], which might indicate reduced mannan biosynthesis. Although the signaling mechanism regulating β-glucan unmasking remains to be elucidated, unmasking of chitin is regulated via the pH-dependent **Rim101** signaling cascade [24] (Fig. 2). The Rim101 signaling cascade has been shown to be involved in the regulation of chitin exposure in other fungal pathogens [69], suggesting that this may be an evolutionary conserved process.

Lactate stimulates enhanced glucan masking

In contrast, it has become clear that host inputs can also stimulate epitope masking. This discovery emerged from the observation that growth of *C. albicans* cells on carbon sources such as amino, fatty or carboxylic acids, rather than sugars, leads to changes in their cell wall architecture and elasticity [65, 70] which affect host immune responses [21]. Further investigation revealed that *C. albicans* cells trigger masking of the PAMP β-glucan at their cell surface in response to physiological levels of lactate, generated either by host or bacterial cells [19] (Fig. 2). This lactate-induced β-glucan masking correlates with a reduction in neutrophil recruitment and an attenuation of inflammatory responses [19]. No doubt these effects contribute to the impact of carbon source adaptation upon the virulence of *C. albicans* during systemic and mucosal infection [65].

Lactate-induced β-glucan masking is mediated by a specific signaling pathway that appears to have recruited key components from other well-characterised signaling pathways. Lactate is detected by the receptor, **Gpr1p** [19], which lies upstream of the cyclic AMP-protein kinase A

pathway that activates hyphal development [71] (Fig. 2). The lactate signal is then transduced to the transcription factor **Crz1p** [19]. Crz1p, which regulates cell wall remodeling, also responds to calcium ions via calcineurin signaling [72]. The mechanisms by which Gpr1p regulates Crz1p activity, and by which Crz1p drives β-glucan masking, are under investigation.

Immune attack triggers β-glucan unmasking

Clearly, *C. albicans* cells can tune the exposure of key epitopes at their cell surface to specific signals in host microenvironments. Interestingly, β-glucan becomes unmasked naturally over the course of disseminated *C. albicans* infection *in vivo* [29]. Recent work demonstrates that this PAMP unmasking is an active fungal response to attack from host neutrophils. Intriguingly, the fungal response also includes the deposition of chitin, which co-localizes with β-glucan unmasking [22] (Fig. 3). As expected, this altered pattern recognition following neutrophil extracellular trap (**NET**)-mediated cell wall changes enhances the subsequent responses of macrophages to damaged fungi. Importantly, neutrophils are required for the time-dependent unmasking of β-glucan *in vivo*, suggesting that these cell wall integrity pathways responding to immune attack *in vitro* are active during infection [22].

The signaling and machinery involved in this fungal response have been partially elucidated (Fig. 3). Chitin deposition and β-glucan unmasking are both regulated by **Hog1p** signaling. Chitin synthase 3 (**Chs3p**) localizes to attack sites and is responsible for the majority of the chitin deposition [22]. In contrast, the stress-activated synthases **Chs2p** and **Chs8p** are not required for this immune-triggered remodeling, highlighting differences in responses to abiotic and biotic stress [73]. Two other proteins associated with cellular and cell wall architecture, Phr1p and Sur7p, are recruited to immune attack sites with unique temporal dynamics, which suggests there is well-choreographed remodeling response to host immune action. These experiments thus give us a glimpse of key immune and cell wall integrity processes that are likely to act following immune attack during infection.

Why is fungal epitope modulated during infection? Can we leverage this to enhance immunity?

Despite the foundation of knowledge outlined above, the role of fungal cell wall remodeling in adapting to the complex host environment and interactions with immunity remains poorly

understood. Unmasking epitopes could be beneficial to the host, as it subverts attempts at immune evasion and enhances host responses directed against the pathogen. This appears to be the case for β-glucan [19, 22, 24, 34, 62] and chitin [27, 74]. Given that so many types of fungal pathogens mask β-glucan and other epitopes, there is a strong argument that blocking this masking benefits the host. Alternatively, in other host contexts, dynamic fungal cell wall remodeling could directly benefit the pathogen by reinforcing the cell wall [22, 28, 75] or exacerbating immunopathology [22, 25, 76-81].

Clearly, the fungal cell wall is a nexus of physiology, drug action and immunity. Therefore, an understanding of how the fungal cell wall senses, responds and remodels itself in response to stress can be leveraged on two fronts. First, this work will identify the fungal pathways critical for adapting and maintaining cell wall integrity during interactions with the host and immunity, potentially yielding novel targets for broad-spectrum antifungal drugs. Second, understanding how dynamic cell wall remodeling, especially epitope unmasking, influences host immune responses to fungi will open the door to designing novel immunotherapeutic strategies for patients with fungal infections.

Figure Legends

Figure 1. Stress-triggered cell wall remodeling affects immune recognition through epitope (un)masking. Cellular stresses, including pH, hypoxia, altered carbon sources, and immune attack can trigger fungal response pathways that remodel the cell wall and alter PAMP availability. The effects on the cell wall lead to enhanced exposure or increased masking of cell wall β-glucan and chitin. Changes in the availability of these PAMPs alter immune recognition and responses.

Figure 2. Abiotic environmental conditions can either enhance PAMP exposure or lead to greater masking. (A) Schematic outlining how acidic pH and lactate signal *C. albicans* to alter availability of β-glucan and chitin for immune recognition, based on [24] and [19]. (B) *C. albicans* cells exposed to glucose or lactate were stained for exposure of β-glucan (Fc-Dectin-1, green), mannan (Concanavalin A, red) and chitin (wheat germ agglutinin, blue). Image generated by Gabriela Avelar, Aberdeen Fungal Group. (C)) *C. albicans* cells exposed to growth media buffered to pH 6 or pH 4 were stained for exposure of β-glucan (Fc-Dectin1, green) chitin (wheat

germ agglutinin, cyan) and total chitin (Calcofluor white, magenta). Arrowheads indicate points of β-glucan exposure.

Figure 3. Immune-mediated attack of fungal hyphae triggers epitope exposure. (A) Neutrophil attack through extracellular trap (NET) formation, including activity of phagocyte oxidase, myeloperoxidase (MPO), and reactive oxygen species (ROS) causes cell wall protein damage and provokes stress responses. (B) Cell wall remodeling triggered by NET attack includes localized Chs3p-mediated chitin deposition and enhanced β-glucan exposure. Unmasking of β-glucan is reduced or abolished in the absence of Hog1p or Chs3p activity. The Phr1p transglycosyltransferase localizes to sites of neutrophil attack and may actively remodel β-glucan in the cell wall. Indirect effects are indicated by dotted lines. Schematic based on [22].

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463

Trends Box

- The fungal cell wall, which consists of proteins and carbohydrates like β-glucans and chitin, is a dynamic organelle that provides structure, protects fungal viability and controls interactions with the host during infection. Fungal cell wall architecture allows opportunistic fungal pathogens to evade innate immune recognition.
- Immune attack and environmental stresses drive cell wall remodeling that unmasks or covers up cell wall epitopes and alters immunogenicity
- Alternative carbon sources and acidic pH present in vulvovaginal candidiasis infections have distinct effects on epitope masking. pH is sensed by Rim101p and the presence of lactate is sensed through Gpr1p and Crz1p.
- Neutrophil attack on *C. albicans* hyphae triggers chitin deposition and enhanced recognition of cell wall β-glucan. This is sensed through the Hog1p MAP kinase, and changes are effected through the chitin synthase Chs3p.
- Altered innate immune recognition due to stress during infection may affect immune and disease dynamics. Components of the pathways that drive these changes therefore represent potential targets for small-molecule interventions.

Outstanding questions box

- Can we enhance fungal clearance by targeting components of pathways that regulate epitope exposure?
- Are stress-induced changes to the cell wall seen in murine infection and *in vitro* actually relevant for human infections? What is the end result when multiple stresses are present?
- How relevant are the *C. albicans* stress response pathways for other human fungal pathogens such as *Aspergillus fumigatus*, *Cryptococcus neoformans*, *Pneumocystis* and dimorphic fungi?
- Through which intermediates does stress sensing lead to changes in the cell wall?
- How is cell wall remodeling spatially restricted to repair localized areas of damage?
- What cell wall stresses are felt during infection in different tissues? Where and when are they felt? How are they sensed?
- Fungal cell wall remodeling can have dramatic impacts on interactions with the host. Does epitope unmasking have beneficial or detrimental impacts on the host during infection?
- Are responses to cell wall stresses dictated by adaptation to host niches of different fungal pathogens? Which fungal responses are conserved throughout the host and which are niche-specific?

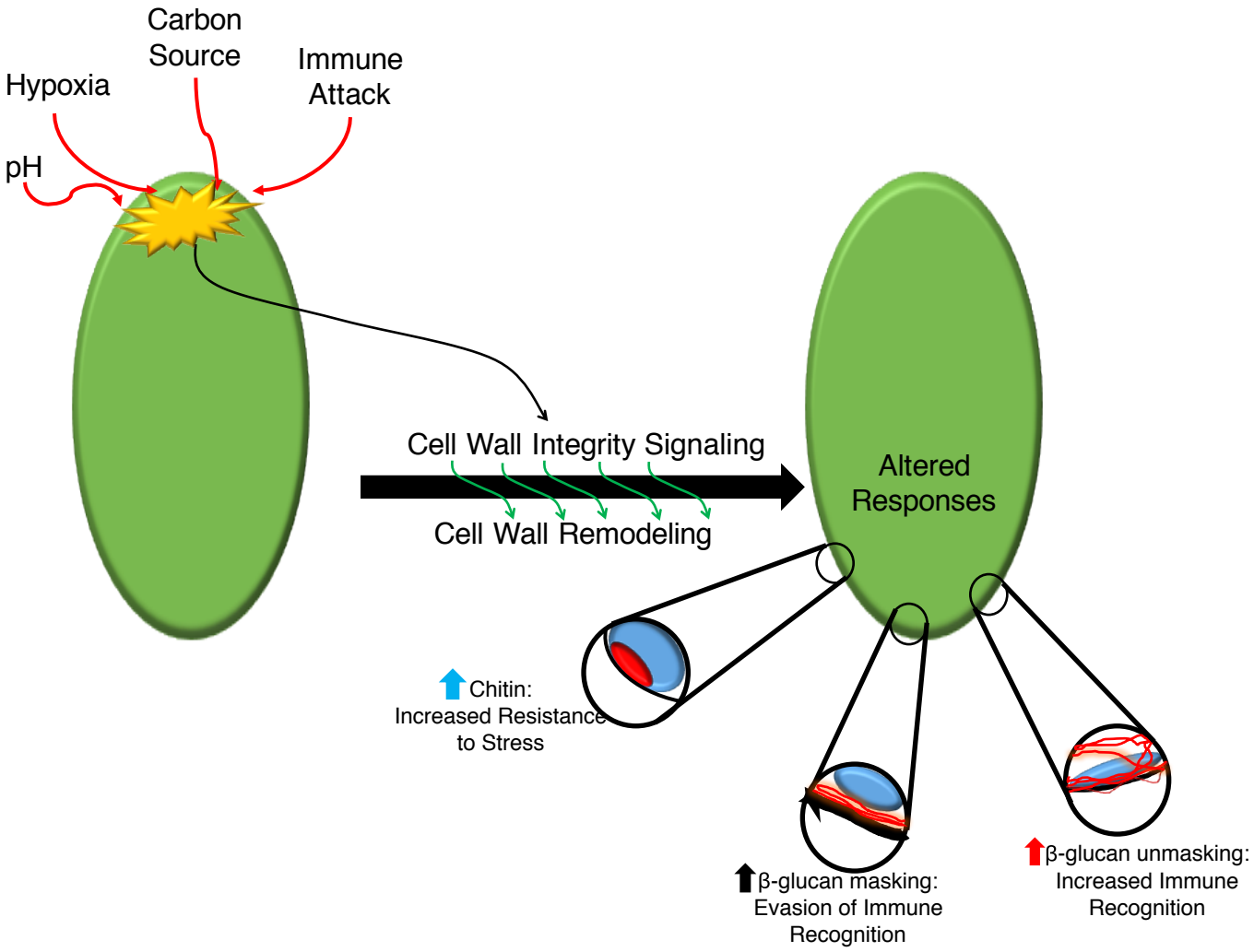
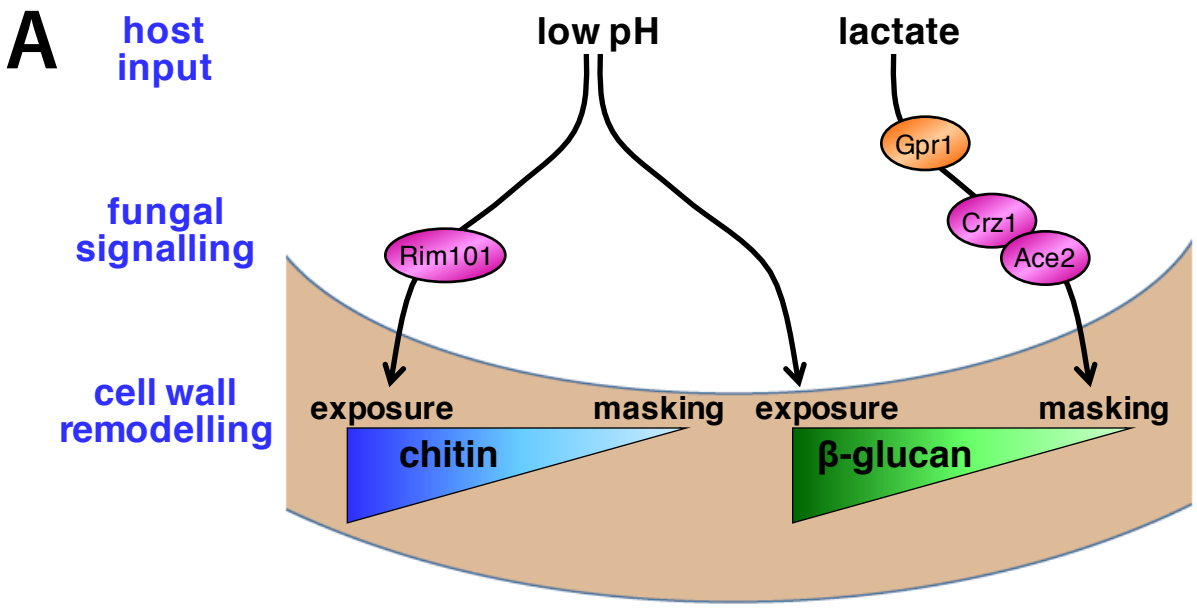


Figure 1


A
host input
low pH
lactate
Gpr1
fungal signalling
Crz1
Ace2
Rim101
cell wall remodelling
exposure
masking
exposure
masking
chitin
β-glucan

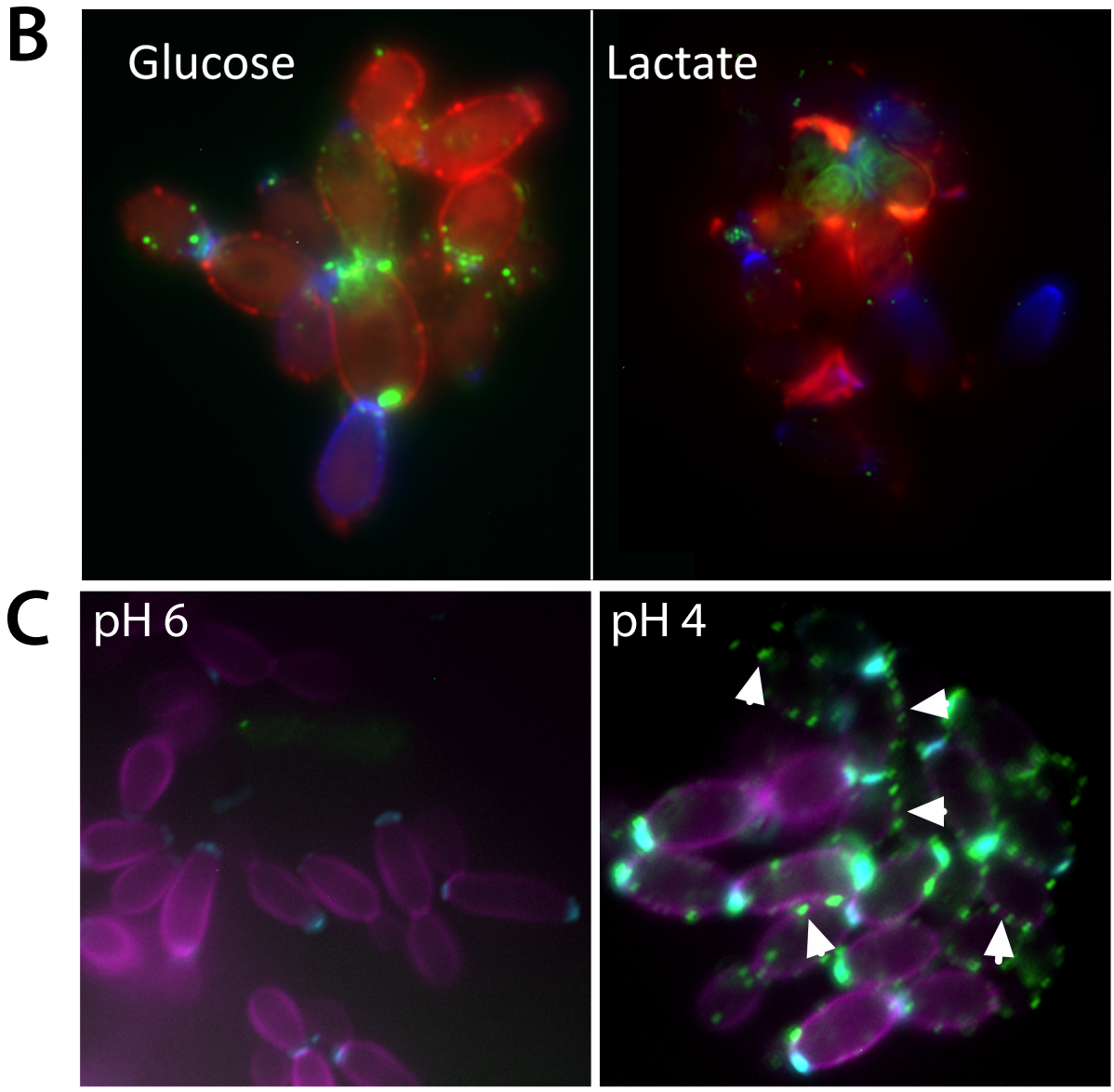

B
Glucose
Lactate
C
pH 6
pH 4

Figure 2

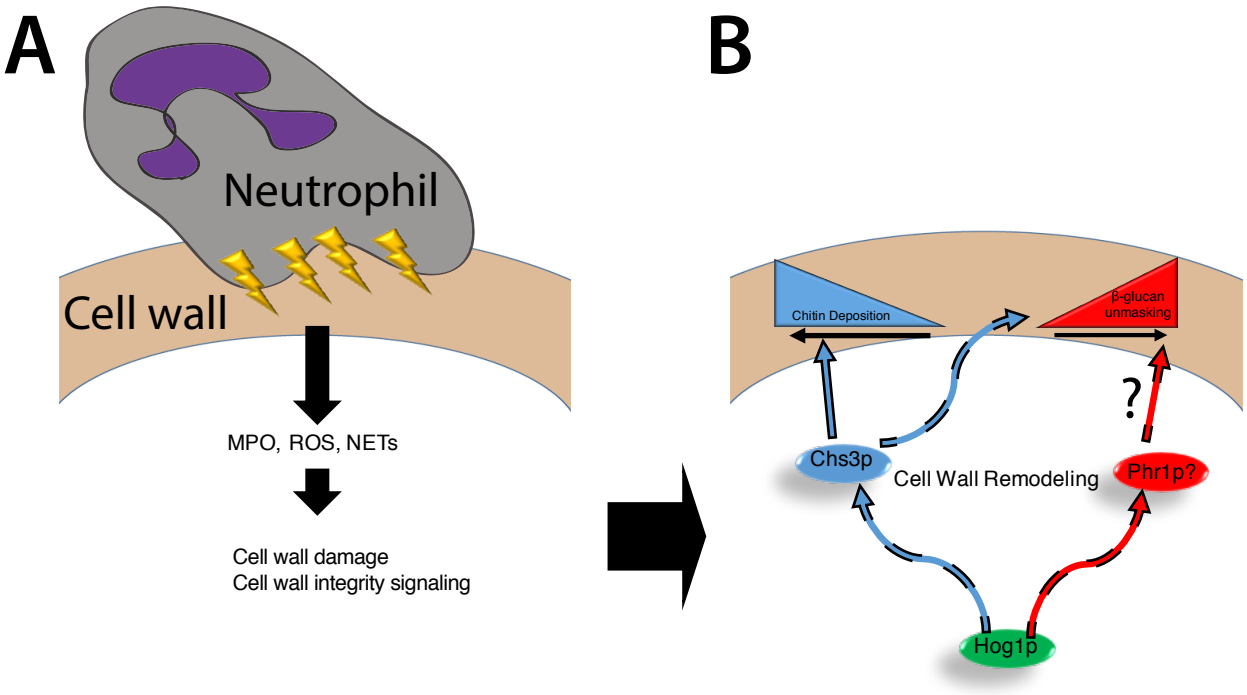

A
Neutrophil
Cell wall
MPO, ROS, NETs
Cell wall damage
Cell wall integrity signaling
B
Chitin Deposition
β-glucan unmasking
?
Chs3p
Cell Wall Remodeling
Phr1p?
Hog1p

Figure 3